The shape of porcine neural progenitor cell cellular genealogies revealed by time-lapse imagining

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as donor cells, and to characterise the derived putative nuclear transfer ES (ntES) cells for their stemness. Efficiency of the donor cells for nuclear transfer was also compared, and an overall cleavage and morula formation rates of 62.44 ± 3.9% and 35.30 ± 3.86%, 75.45 ± 3.92% and 45.84 ± 3.86%, and 56.38 ± 3.92% and 29.09 ± 3.86% were obtained from adult fibroblasts, ES cells, and lymphocytes, respectively. A significant difference was found between ES cells and the other 2 donor cells in terms of cleavage and morula formation. However, no such difference existed between fibroblasts and lymphocyte donor cells. Stem cell colonies were successfully derived from HMC embryos obtained from all 3 different donor cells. The rate of primary colony formation was 61.66 ± 4.62% for fibroblast-donor-cell-derived embryos. This rate was 59.91 ± 4.62% for ES-donor-cell-derived embryos and 62.49 ± 4.62% for lymphocyte-donor-cell-derived embryos. The putative ntES colonies were positively characterised for *TRA-1-60, TRA-1-81, SSEA-1, SSEA-4, OCT-4, SOX-2*, and *Nanog* by immunocytochemistry and RT-PCR. Results indicated that ES cells had better efficiency as donor cells in cloned embryo production than did adult fibroblasts and lymphocytes. The finding also suggested that terminally differentiated cell-like lymphocytes can also be reprogrammed. Moreover, there was no difference between the different donor-cell-derived HMC embryos in terms of ntES cell derivation. The study has established an efficient protocol for putative ntES cell derivation from HMC embryos. This could be of substantial significance because patient-specific ntES cells have proven therapeutic significance.

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295 THE SHAPE OF PORCINE NEURAL PROGENITOR CELL CELLULAR GENEALOGIES REVEALED BY TIME-LAPSE IMAGING

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Porcine neural progenitor cells (pNPC) derived from embryonic stem cells are capable of self-renewal and differentiation into neural and glia lineages, rendering them promising candidates for cell-based therapy of neurodegenerative diseases in a large animal biomedical model. A prerequisite for the successful future therapeutic use of pNPC is a comprehensive characterisation and understanding of the neurogenic process. This is important for learning how to direct cell fates into required proportions of the cell type wanted for the specific brain disease to be treated, and it is crucial for avoiding uncontrolled cell proliferation leading to fatal tumour formations. Time-lapse analysis is a powerful tool to obtain live cell characterisation by analysing individual cell fate. Information on cellular development, division, and differentiation can be composed into a pedigree-like structure denoted as cellular genealogy giving an overview of the proliferation profile of a cell culture and the duration of each cell cycle (Al-Kofani *et al.* 2006). The aim of the study was to construct cellular genealogies of pNPC and differentiated neural lineages, respectively, by time-lapse imaging to evaluate the effect of external variables observed by changes in the topology of the cellular genealogy. Porcine NPC were derived from epiblast cells isolated from day-9 porcine blastocysts and cultured in DMEM/12, Pen/strep, B27 and N2 with basic fibroblast growth factor and epidermal growth factor, and differentiation was obtained by withdrawal of basic fibroblast growth factor and epidermal growth factor. The state of cellular development of undifferentiated and differentiated pNPC was verified immunohistochemically by the presence of *SOX2, NESTIN, TUJI*, and *GFAB* (Rasmussen *et al.* 2010). The time-lapse images were captured by a Nikon Biostation with a 10× resolution under phase contrast in a humidified chamber at 38°C with 5% CO_2, 5% O_2, and 90% N_2. For each sequence, images were captured at intervals of 10 min in 16 frames. Sequences 1, 2, and 3 constituted passage 15 pNPC, passage 4 pNPC, and presumably differentiated cells, respectively. For each sequence, cell cycle length was calculated after manual tracking of selected cells. The cell cycle length of pNPC is shown in Table 1. Based on these data, cellular genealogies characteristic of each individual cell type have been constructed.

Table 1. Cell cycle length of porcine neutral progenitor cells (pNPC) before and after differentiation

Type of cell	Cell cycle length		
	1st cycle	2nd cycle	3rd cycle
Passage 15 pNPC	40.7 ± 6.6 ($n = 3$)	23.6 ± 3.1 ($n = 5$)	No data
Passage 4 pNPC	39.2 ± 5.7 ($n = 2$)	13.8 ± 3.2 ($n = 2$)	20.4 ± 6.2 ($n = 2$)
Passage 4 differentiated	46.0 ($n = 1$)	63.3 ($n = 1$)	No data

296 PRODUCTION OF HEMIZYGOUS AND HOMOZYGOUS EMBRYONIC STEM CELL-DERIVED NEURAL PROGENITOR CELLS FROM THE TRANSGENIC ALZHEIMER GÖTTINGEN MINIPIG

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Alzheimer's disease is the most prevalent cause of dementia and afflicts ~26 million people worldwide. There are currently no cures for this disease. Production of *in vitro* models of the disease would be extremely useful for studying disease mechanisms and for potential screening of novel drugs. In this study we produced 2 hemizygote and 2 homozygote embryonic stem cell-derived neural progenitor cell lines from Day 8 transgenic blastocysts carrying a human gene linked to early-onset Alzheimer's disease [Swedish mutation of the amyloid precursor protein (hAPPsw)]. Following onset of

spontaneous oestrus, a mating of hAPPsw$^{\pm}$ × hAPPsw$^{\pm}$ Göttingen transgenic progeny was performed. Eight days after the first of 2 matings, embryos were flushed from the tip of both cornuas of the gilt under surgical anaesthesia. A total of 6 blastocysts were obtained and 7 corpora lutei recorded. Blastocysts were transported for 4 h in porcine zygote medium 3 (PZM-3) in hypoxic, humidified conditions at 39°C to the cell laboratory. Compact epiblasts were mechanically isolated from the embryo using insulin needles and cultured on inactivated mouse embryonic fibroblasts in embryonic stem cell medium, supplemented with 20 ng mL^{-1} human recombinant basic fibroblast growth factor (Prospec) and 20 ng mL^{-1} human recombinant Activin A (Prospec), for a period of 5 days in hypoxic conditions at 39°C. Five of the 6 epiblasts expanded to form embryonic stem-cell-like outgrowth colonies. These were cut into small colonies and plated on MS5 murine stromal cells to induce spontaneous neural differentiation in DMEM medium containing 15% knockout serum replacement. Neuronal rosette-like structures were identified from Day 10 of differentiation onward. Six rosette structures were mechanically isolated from 4 outgrowths and plated in serum-free conditions on Matrigel-coated dishes. Two of the 6 lines failed to proliferate beyond passage 2. The 4 remaining cell lines have currently been cultured to passage 7. These lines were analysed at passage 5 by comparative real-time PCR and found to be positive for the neural progenitor markers *VIMENTIN, SOX2, NESTIN PAX6, MUSASHI*; other neural markers *BETAIIITUBULIN* and *NCAM*; and the astrocyte marker, *GFAP*. These lines were also subjected to analysis by immunocytochemistry and found to express SOX2, VIMENTIN, and NESTIN. Further genotyping by comparative real-time PCR using primers designed to target the hAPPsw gene revealed that 2 lines carried a single copy of hAPPsw and 2 lines carried 2 copies of hAPPsw. The expression levels of the hAPPsw transgene in these cell lines were determined using quantitative PCR. These cell lines are currently being investigated for their ability to differentiate into cholinergic neurons and for their expression of hyperphosphorylated TAU and β-Amyloid secretion. These cell lines will be potentially relevant for the *in vitro* study of amyloid precursor protein accumulation in neural cells and its role in cell death, as well as for potential screening of novel drugs for Alzheimer's disease.

297 DERIVATION AND CHARACTERIZATION OF THE TRANSGENIC SOMATIC CELL NUCLEAR TRANSFER-DERIVED BOVINE EMBRYONIC STEM CELLS

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Bovine transgenic embryonic stem (ES) cells have not been reported yet because it seems that the derivation methods and the culture conditions for the inner cell mass are neither consistent nor optimized. Isolation of inner cell mass and primary culture of ES colonies is a critical step toward the establishment of authentic bovine ES cell lines. Herein, we reconstructed somatic cell nuclear transferred (SCNT) bovine blastocysts carrying a vector expressing the human *INF-α* gene, and isolated inner cell masses to derive transgenic bovine embryonic stem cells. In addition, we added 2 inhibitors, inhibition (2i system) of the mitogen-activated protein kinase (Erk1/2) cascade, PD0325901(3 Î1/4M), and of glycogen synthase kinase 3, CHIR99021 (1 Î1/4M), in the inner cell mass primary culture to check reliability of the 2i system for bovine ES culture. The 2 inhibitors made the morphology of colonies more intact, and primary colonies were better maintained in early passages. However, there were no significant effects on the attachment rate and maintenance in late passages (percent of percent over 3 passages: 2i system, 21/38 (55.3%); control, 22/42 (33.3%); $P < 0.05$). Inner cell masses were isolated mechanically and subcultured by an enzymatic in primary inner cell mass culture. Massive growth of trophoblast cells appears to inhibit inner cell mass growth, so hatching and hatched blastocysts were cut with a needle to remove trophoblast cells. Poor quality blastocysts were attached by the whole seeding method, and the margin trophoblast cells were consecutively removed in early passages. Established bovine ES cells express alkaline phosphatase, *Oct-4, SSEA1, SSEA4, Tra-1–60*, and *Tra-1–81*. We confirmed pluripotent gene expression of bovine ES like cells; *Oct-4, SSEA1*, and *Rex 1* were positive, but trophoblast marker *CDX2* was negative. This study shows that the 2i system is a reasonable method for use during inner cell mass culture in early passages. We established 6 transgenic nuclear transfer bovine ES cell lines with the 2i system and 4 *in vitro* fertilized bovine ES cell lines (all were over 10 passages).

298 DIFFERENTIATION OF CANINE AMNIOTIC FLUID MESENCHYMAL STEM CELLS INTO NEURONAL PRECURSORS

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The amniotic fluids contain mesenchymal stem cells and can be readily available for tissue engineering. Recently, regenerative treatments such as tissue engineering, cell therapy, and transplantation have shown potential in clinical trials of degenerative diseases. Physiologically, disease presentation and clinical responses in the dog are much more similar to that in the human compared with other traditional mammalian models. In addition, several researchers have demonstrated *Canis familiaris* is a suitable model for human diseases. The aim of the present study was to investigate whether canine amniotic fluid (cAF)-derived mesenchymal stem cells (MSC) can differentiate into neural precursor cells *in vitro* by neural induction reagent. The conditions of differentiation of MSC into neural cells were DMEM and N2-supplement, dibutyryl cyclic adenosine monophosphate, and butylated hydroxyanisole. During neural precursor differentiation, cAF-MSC can progressively acquire neuron-like morphology. Expressions of neuron cell-specific markers were examined before and after *in vitro* induction of differentiation. Changes in mRNA levels of specific genes were quantified by RT-PCR. The mRNA levels of *NEFL* (730%), *GFAP* (350%), β-tubuline 3 (2900%), and *NSE* (960%) were significantly increased after induction. The value of change in mRNA levels before and after induction was evaluated with the Image J program. In addition, the nestin, β-tubuline 3, and tyrosine hydroxylase protein expressions were confirmed by immunocytochemistry assay following the induction of differentiation, compared with the noninduction. In conclusion, this study demonstrated that cAF-MSC have great potential for neural